STRUCTURAL STUDIES BY NMR OF SELECTIVELY ELECTROCHEMICAL MODIFICATION OF PROTEINS: TYROSINE NITRATION OF HEN EGG WHITE LYSOZYME

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1.Introduction

The increasing evidence of the relationship between some heart and pulmonary diseases and the presence of nitrated proteins *in vivo* makes the nitration of proteins a biomarker of illness [1], particularly in the case of nitrotyrosine. These alterations affect the metabolic function of proteins, causing cellular dysfunctions [2].

The electrochemical nitration of residues in proteins offers selective production of novel proteins in contrast with traditional methodologies such as protein engineering and the use of chemical reagents. The use of carbonaceous materials such as boron doped diamond (BDD) in the study of the electrochemical nitration of hen egg white lysozyme (HEWL) overcomes the drawback of activity lost, observed when metallic electrodes such as copper or platinum [3,4] are used, thereby unwanted interactions protein-electrode occur. By the use of BDD electrodes, the aim of this communication is to investigate the production of electrosynthetically nitrated tyrosine residues in proteins at BDD electrodes, using lysozyme as a model protein, and to correlate enzyme function and conformational change either by by activity assay and NMR studies respectively.

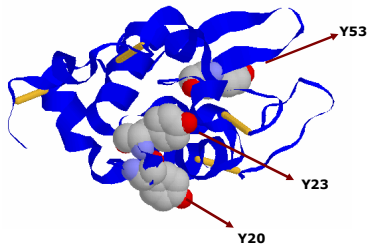


Fig.1 HEWL, 14.3 kDa, 129 amino acids. The most sensitive residues upon electrochemical nitration are tyrosines, but only Y23 in a first step, and later Y20 are nitrated. Y53 remains buried and hence less accessible.

2. Experimental

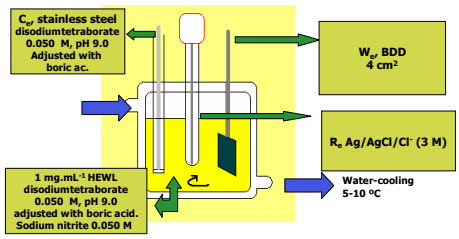


Fig. 2 Scheme of the electrochemical cell used in the reaction at 1.050 V vs Ag/AgCl. A non-selective membrane of fritted glass separates the cathodic compartment of the anodic one. Electrosynthetic nitration is monitorized measuring the charge passed and the absorbance of protein solution at 430 nm, 420 nm and 550 nm. (Molar extinction coefficient of nitrotyrosine is 4,400 $M^{-1}cm^{-1}$ at 430 nm and pH = 10.0)

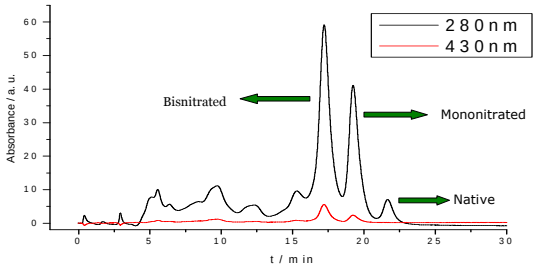
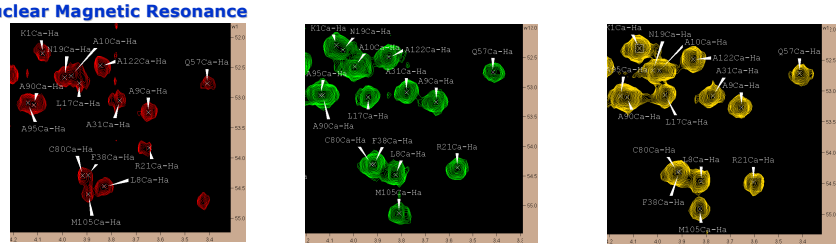


Fig. 3 The products of reaction were separated by preparative chromatography using a strong ionic exchange column (IEC SP-825, 8.0 mm ID x 75 mm length). The incorporation of nitro group into the aromatic ring of tyrosine residues decreases the pK_a of the residue from 10.5 to 7.2. This has important consequences in the lowering of lysozyme isoelectric point.

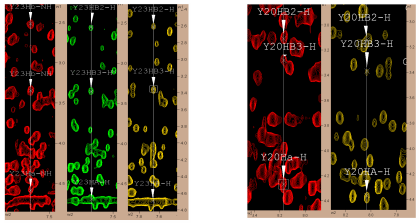
3. Discussion

3.1. Nuclear Magnetic Resonance



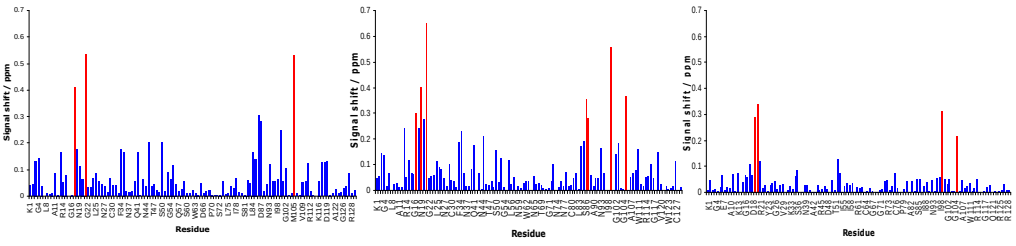
a) b) c)

Fig. 4 Region of the NMR spectra corresponding to heteronuclear correlation experiments to one bond (2D-HSQC), performed at 308 K, at pH 3.8 (adjusted with HCl), ca. 10 % D_2O, working with natural ^{13}C abundance and concentration around 2 mM, using a 500 MHz Bruker spectrometer, for the different purified nitrated lysozymes: a) native b) mononitrated c) bisnitrated.



a) b)

Fig. 6 Regions of the spectra corresponding to TOCSY experiments for the three purified fraction of proteins, pointing out a) Y23 spin system and b) Y20 spin system. The samples were prepared as shown in Fig. 5 and the experiments were carried out at 308 K using a 500 MHz Bruker and a mixing time of 50 ms.



a) b) c)

Fig. 5 Differences in the chemical shifts of the C^{α} in the 2D-HSQC experiments for the different nitrated lysozymes purified by low pressure chromatography. Comparison between a) native and mononitrated lysozyme b) native and bisnitrated lysozyme c) bisnitrated and mononitrated lysozyme. The most remarkable changes occur to those residues by the nitrated tyrosine in the amino acid sequence as well as to those present in the nearest helix opposite the loop (V99 and M105).

	NOESY cosspeaks involving Y20	NOESY cosspeaks involving Y23
Native	Y20H-R21H, Y20H-G22HA3, Y20H-R21HA, Y20H-V99HG2, V99H-Y20HB2, W28H-Y20HB2, R21H-Y20HB3, L17H-Y20HB3	Y23H-R21HB2, Y23H-R21HG, Y23H-L25HB, Y23H-G22HA2, Y23H-V99HG, Y23H-M105HG, Y23H-N103HB2, V99H- Y23HA, W111H-Y23HA, G22H-Y23HB2, G104H-Y23HB2, W111H-Y23HB3
Mononitrated	Y20H-N19HB, Y20H-R21HA, Y20H-R21H, Y20H-V99HB, Y20H-V99HG2, G22H-Y20HB3, L17H-Y20HB3	Y23H-N103HB2, Y23H-R21HG, Y23H-G22HA2, Y23H-L25HE, Y23H-V99HG, Y23H-G22H3, Y23H-M105HG, Y23H-G104HA2, Y23H-N19HB2,G22H-Y23HB2, G104H-Y23HB2, N19H-Y23HB2, M105-Y23HB2
Bisnitrated	Y20H-N19HA, Y20H-V99HA, Y20H-V99HG2, Y20H-N19H, N19H-Y20HA, V99H-Y20HB2	Y23H-V99HB, Y23H-R21HB2, Y23H-G22HA2, Y23H-V99HA, Y23H-G22H3, Y23H-M105HA,Y23H-G104HA2, W28H-Y23HB2,Y20H-23HB2, N19H-Y23HB2

Fig. 7 Crosspeaks corresponding to NOESY spectra at 308 K, and a mixing time of 120 ms for the modified and unmodified tyrosines Y20 and Y23 respectively. Signals regarding the native protein are highlighted in blue. Samples were prepared as shown in Fig. 5 and all experiments were carried with a 500 MHz Bruker spectrometer.

3.2. Activity assay

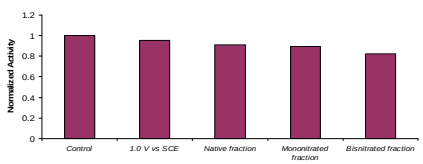


Fig. 8 Cell wall lytic assay using Micrococus Lysodeikticus (Luteus) for the activity measurement of different nitrated lysozymes as described in Fig.2 (0.52 mol of nitrotyrosine per mol of lysozyme). Native lysozyme exposed to the electrode together with those nitrated ones were, after HPLC separation, dialyzed and freeze-dried for further activity measurement.

4. Conclusions

The electrochemical nitration of HEWL at boron doped diamond electrodes provides selectively the mono and bisnitrated lysozyme at Y23 (mononitration) and at Y23 and Y20 (bisnitration). Mass spectrometry analyses demostrated that a mass increase of +45 and +90 occurs, without any other change in the protein.

HSQC spectra C^{α}-H^{α} show slight variations towards signals regarding amino acids closer to nitrotyrosines relative to 3D-structure, such as R21, V99 or M105. This might involve little remarkable changes upon the 3D-structure of the protein. Consequently, TOCSY spectra depict few changes, except those shown by the modification of tyrosines. With respect to NOESY spectra, it is observed that the crosspeak pattern of all three spectra is not identical, hence, it may exist some differences between amino acids of that region towards that of the native structure. The later statment is a consequence of the enhancement of hidrofilicity and polarity due to the incorporation of a NO_2 group into the aromatic ring. Future experiments are on-going about the conformational changes and its modeling by computatinal procedures

Remarkably the lytic activity of enzyme remains unaltered after modification. The use of BDD electrodes shows novel production of modified proteins with applications in labelling, biosensors and for studies in pathophysiological dysfunctions.

5. References

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6. Acknowledgment

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